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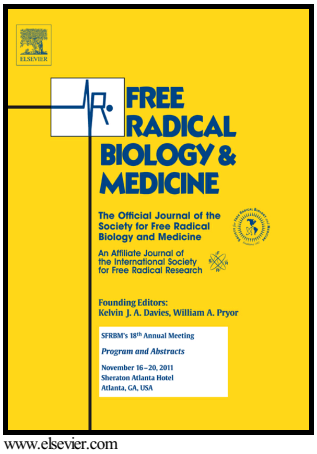

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Diabetic nephropathy: is there a role for oxidative stress?

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Abstract

Oxidative stress has been implicated in the pathophysiology of diabetic nephropathy. Studies in experimental animal models of diabetes strongly implicate oxidant species as a major determinant in the pathophysiology of diabetic kidney disease.

The translation, in the clinical setting, of these concepts have been quite disappointing, and new theories have challenged the concepts that oxidative stress *per se* plays a role in the pathophysiology of diabetic kidney disease.

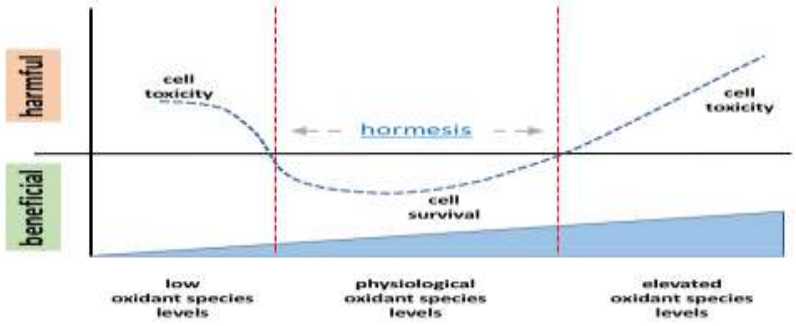
The concept of mitochondrial hormesis has been introduced to explain this apparent disconnect. Hormesis is intended as any cellular process that exhibits a biphasic response to exposure to increasing amounts of a substance or condition: specifically, in diabetic kidney disease, oxidant species may represent, at determined concentration, an essential and potentially protective factor.

It could be postulated that excessive production or inhibition of oxidant species formation might result in an adverse phenotype.

This review discusses the evidence underlying these two apparent contradicting concepts, with the aim to propose and speculate on potential mechanisms underlying the role of oxidant species in the pathophysiology of diabetic nephropathy and possibly open future more efficient therapies to be tested in the clinical settings.

Graphical abstract

Mitochondrial hormesis could represent an important concept that could allow us to dissect the hazardous *vs* beneficial role of reactive oxygen species levels in the pathophysiology of diabetic nephropathy.



Keywords

Diabetes, kidney, oxidative stress

Introduction

Diabetes Mellitus

Diabetes mellitus (DM) is a metabolic syndrome, characterised by chronic hyperglycaemia and glucose intolerance because of defects in insulin action or secretion, or a combination of both [1]. The worldwide burden of diabetes is rapidly rising, with a dramatic increase in prevalence in recent decades [2, 3]. Estimated figures from the International Diabetes Federation predicted that 422 million people were living with diabetes in 2014, and these figures are expected to increase to 642 million by 2040 [4](http://www.diabetesatlas.org). This translates into the reality that a shocking one out of ten people will have diabetes in 20 years. At present, China has been reported to have the greatest incidence of DM, affecting 94.8 million of the population, followed closely by India and the USA [5].

Diabetes is the 5th leading cause of morbidity and mortality worldwide [6]. The significant global impact of this condition is acknowledged internationally, with diabetes ranking highly on the international health agenda [7]. With 5% of the UK population affected by this condition, diabetes has become a major challenge in 21st century healthcare [8, 9]. This is largely due to the associated micro and macro-vascular complications of diabetes, affecting diabetic populations in both developed and developing countries [7, 9]. The microvascular complications include diabetic nephropathy, retinopathy and neuropathy, and are responsible for significant morbidity [10]. The diabetic macro-vascular complications represent the most common cause of mortality in patients with diabetes; these include accelerated coronary heart disease, ischemic stroke and peripheral vascular disease [11].

Classification of Diabetes Mellitus

The American Diabetes Association classification of DM is currently the most widely used classification system for diabetes in clinical practice [12]. Classification of DM is largely based on aetiology and not pharmacologic treatment, largely due to an improved understanding of the causes underlying diabetes. The two major forms of diabetes are type 1 diabetes mellitus (T1DM), an auto-immune disorder characterised by the destruction of the insulin producing β-cells in the islets of Langerhans which accounts for 5-10% of cases, and type 2 diabetes mellitus (T2DM), a more common form of DM which accounts for up to 90% of cases and is due to impaired insulin secretion and/or insulin action, as a result of insulin resistance [12]. With the increasing global incidence of obesity, T2DM now typically affects a younger and more obese patient group [7].

Diabetic Nephropathy

Classification of Diabetic Nephropathy

Diabetic nephropathy (DN), a serious and most feared microvascular complication of diabetes, is a chronic progressive disease of the kidney, characterised by persistent albuminuria and progressive

relentless decline in glomerular filtration rate (GFR). Diabetes leads to disruption of the renal microvasculature, with progressive damage at the level of the glomerular capillaries and tubular interstitium [13]. Traditionally, DN has five identified stages based on GFR and urinary albumin excretion (UAE): glomerular hyperfiltration, a silent stage, incipient nephropathy with microalbuminuria, overt nephropathy and, finally, end stage renal disease (ESRD)(**Table 1**)[14].

Diabetic nephropathy is currently the leading cause of ESRD in the western world. Consequently, diabetes is a principal cause for patients requiring renal replacement therapy, with diabetic patients contributing to approximately 45% of the individuals on renal replacement therapy worldwide [15].

Epidemiology of Diabetic Nephropathy

The incidence of DN has substantiality increased over recent years, although recent data from the United States Renal Data System (USRDS) suggests that incidence counts for ESRD are beginning to plateau, whilst prevalence counts are rising [16]. DN develops over time, with a peak incidence after 10-20 years duration of diabetes and affects a striking 45% of diabetics, with a similar incidence in both T1DM and T2DM patients [13]. In some patients the diabetes-driven renal damage is so minimal that it remains clinically silent during their entire lifetime [17]. Importantly, following 5 years of consistent proteinuria, the cumulative risk of developing ESRD in both T1DM and T2DM stands an estimate of 60% [18]. Ethnicity can influence the severity and incidence of DN, as DN is more prevalent in African Americans, Asians and Native Americans, compared to White Caucasians populations [19].

Cardiovascular-Renal Complications of Diabetic Nephropathy

Diabetes increases the risk of developing cardiovascular disease (CVD) by two-fold compared to the non-diabetic general population. The incidence of myocardial infarction in the diabetic population is increased by three-fold compared with the general population and DN considerably

adds to this observed increase of cardiovascular morbidity and mortality [20, 21]. Furthermore, DN is also a major risk factor for CVD, which is currently the leading cause of death in both T1DM and T2DM worldwide [22-24]. In the diabetic population, CVD accounts for more than half of deaths [25]. A large majority of patients with DN will die even before progression to ESRD as a result of CVD related causes [26]; importantly DN is associated with an increase in myocardial infarction and cerebrovascular events [27]. The significant healthcare burden due to the epidemic of DN, on both the individual and the healthcare system, is of major concern and requires attention to achieve a suitable and effective solution. A better understanding of the pathophysiology underlying DN is crucial to direct methods for early intervention and novel treatments to prevent the progression and reduce cardiovascular morbidity and mortality in the future.

Oxidative stress in the pathogenesis of diabetic nephropathy

Oxidant species are products of normal oxygen metabolism and are important in processes such as cell signalling, ageing and degenerative disease [28]. In health, an intricate balance exists between oxidant species, recognised important signalling molecules for normal cell physiology [29] and anti-oxidant activity to prevent potential damaging effects secondary to excess oxidant species tissue accumulation. Importantly, factors that lead to an imbalance in the oxidant species /anti-oxidant equilibrium, either in the form of increased oxidant species production or diminished anti-oxidant activity, can lead to excess oxidative stress and subsequent tissue injury [28].

Numerous pathophysiological mechanisms underlying DN have been described, in which increased oxidant species have been identified as the single unifying upstream event [30]. Thus, increased oxidant species holds a central and prominent role in the pathophysiology of diabetic microvascular complications, and for this discussion, DN.

In vitro and *in vivo* experimental models of diabetes have established that metabolic (hyperglycaemia, dyslipidaemia)[31] and haemodynamic (systemic and glomerular hypertension) [17] insults represent the two major drivers of oxidative stress in the diabetic kidney.

The kidney is particularly vulnerable to damage caused by elevated circulating glucose levels. The nephron (glomeruli and tubuli) is an insulin independent organ and the flux of glucose into the cells is regulated by the ambient circulating glucose levels and the expression of facilitative glucose transporters (GLUT1)[17].

Indeed, the interaction between metabolic (hyperglycaemia) and haemodynamic (hypertension) perturbations, and their secondary modulation of different intercellular signalling pathways, appears to be key and represents an important driver of DN (**Fig. 1**). Hyperglycaemia-mediated increase in vascular nitric oxide [32] and transforming growth factor (TGF)-β1 [33, 34] through the production of oxidant species [35], have been linked to vasodilation of both afferent and efferent glomerular arteriolae. Hyperglycaemia also activates the local tissue renin-angiotensin-aldosterone system (RAAS): a concept first proposed by Hostetter [17, 36] whereby local (glomerular) dysregulation of the RAAS results in local excess production of angiotensin II. In diabetes, the documented higher sensitivity of the efferent (versus the afferent) glomerular arteriole to the vasoconstrictive action of angiotensin II, contributes to the imbalance in arteriolar tone which then results in higher glomerular capillary pressure [17, 37]. As a result, in diabetes, a disproportionate systemic pressure is transmitted to the glomerular circulation resulting in glomerular cell mechanical elongation and activation of the cellular mechanisms that lead to glomerular damage [38].

The haemodynamic perturbations have been proposed as a potential mechanism for upregulation of the facilitative glucose transporter GLUT1 in mesangial cells, resulting in increased glucose flux into the cells and secondary cell damage [39]. An excess in cellular glucose transport due to metabolic-haemodynamic interaction, synergistically fuels an increase in oxidant species production and the manifestations of DN and other diabetic microvascular diseases [40].

The Glomerular Filtration Barrier (GFB)

DN causes unique ultra-structural alterations in the nephrons of the kidney, at the level of the glomerulus. The GFB is a complex structure, composed of three key components; specialised epithelial cells known as podocytes, highly fenestrated glomerular endothelial cells and the glomerular basement membrane (GBM), a thin membrane separating these two groups of cells. In health, this structure plays an important role in maintaining the permselective function of the glomerulus. The distinct ultrastructure of glomerular endothelial cells, which lack fenestra diaphragms between the fenestrations and are covered by a glycocalyx, facilitates the filtration of water and small solutes and regulates the permeability of the glomerulus in physiology [41, 42]. Albuminuria is a direct result of defects at the level of the GFB [41] and is one of the earliest signs of DN. The degree of albuminuria can correlate with and is also an important clinical predictor of the rate of progression towards ESRD [43, 44]. Moreover, according to the Steno hypothesis, the presence of increasing albuminuria is indicative of widespread systemic vascular damage [45]. Therefore, understanding the normal structure, function and physiology of the glomerulus and the GFB and the changes that occur in DN is crucial. Hallmark pathological changes that occur in DN at the glomerulus include diffuse mesangial expansion and sclerosis, alteration of the endothelial glycocalyx, GBM thickening, podocyte foot process effacement and detachment and a reduction in podocyte number [46](**Fig. 2**).

Mesangial Cells in DN

Mesangial cells hold the glomerular capillaries and form the glomerular tuft (**Fig. 2**). In diabetes, progressive deposition of extracellular matrix sclerosis affects the glomerular tuft initially. In humans, progressive mesangial expansion has been proposed as the main mechanism for loss of kidney function [47]. This progressive extracellular matrix deposition and accumulation results in

the formation of nodules, first described by Kimmelstiel and Wilson, that with progressive matrix deposition lead to capillary obliteration and progressive and diffuse glomerulosclerosis [48].

Glomerular endothelial cells in DN

The endothelium plays a central role in the pathophysiology of diabetic glomerulopathy. Endothelial dysfunction precedes glomerular permeability to albumin [49, 50], and has been suggested as the initial pathogenic mechanism for chronic vascular diabetic complications [45, 51]. Diabetes drives glomerular endothelial cell injury by causing loss of glycocalyx and promoting cell apoptosis [52]. The glycocalyx is composed of heparan sulphates, hyaluronic acid, sialoprotein, and proteoglycans [53, 54], and forms a fluid extracellular layer that covers the glomerular capillary lumen; changes in the glycocalyx results in alteration of glomerular endothelial cell function and alteration in vascular permeability [41, 52].

Podocytes in DN

Podocytes are terminally differentiated glomerular cells with distinct interdigitating primary and secondary foot processes, which are linked to form slit diaphragms [55]. These slit diaphragms are specialised size-selective barriers composed of nephrin, neph1 and podocin proteins which tightly regulate the size of molecules in the glomerular filtrate, preventing the filtration of macromolecules [56, 57]. Nephrin also has an associated role in the maintenance of normal podocyte actin cytoskeleton structure and function through interactions with signalling proteins and cascades, namely phosphoinositide 3-kinase (PI3K), PI3K-dependant Protein kinase B (AKT) phosphorylation and, subsequent, increased Ras-related C3 botulinum toxin substrate-1 (Rac1) activity [58]. In health, podocytes are crucial in preserving the integrity of the glomerular capillaries, regulating the synthesis of extracellular matrix proteins and maintaining the permselectivity of the GFB [59].

In diabetic environments, the structure and function of podocytes are disrupted, leading to excess extracellular matrix deposition, subsequent GBM thickening and foot process fusion and detachment from the GBM [60]. Research has suggested that podocyte damage is often the trigger of the cascade that results in major structural and functional disruptions of the glomerular capillaries in glomerular disease [61]. Evidence from studies looking at the early glomerular alterations in experimental animal models of T2DM in ZDF-fa/fa rats and Goto Kakizaki rats have shown podocyte injury present without mesangial expansion, suggesting the podocytes are the initial trigger in diabetes-induced glomerular disease [62].

Experimental animal models of diabetes have demonstrated that podocyte loss is followed by foot process widening in remaining podocytes, which is believed to be a compensatory mechanism in order to cover the exposed GBM surface area as podocytes lack regenerative capacity and cannot be replaced [63]. This largely results in proteinuria and at a later stage, progressive glomerulosclerosis.

Oxidative stress in the glomerulus

In the glomerular capillaries, oxidant species-mediated damage affects all the layers of the glomerular filtration barrier, beginning with functional alterations of the interaction between glomerular endothelial cells with their glycocalyx layer and podocytes [64], followed by extracellular matrix deposition mainly characterised by an increased expression/secretion of type-IV collagen [65].

Importantly, the endothelial cells glycocalyx, mainly composed by proteoglycans and glycosaminoglycans enriched in heparan sulphate, recognised as a crucial component of the glomerular filtration barrier [66, 67], is a major target for oxidant species. Excess hydrogen peroxide favours shedding of heparan sulphate from glycosaminoglycans and/or glycosaminoglycans degradation, secondary decrease in anionic charges and increase in glomerular permeability to macromolecules [68, 69]. Oxidant and nitrogen species-mediated

activation of matrix metalloproteinases and inhibition of endogenous protease inhibitors has also been proposed as a potential mechanism for glycocalyx degradation [70, 71].

Similarly, the GBM, known to retain charge properties for the anionic heparan sulphate side chains attached to the core proteins agrin and perlecan, and the fine extracellular matrix network structure, crucial in maintaining its permselective properties [72], can be targets of excess oxidant species production. Hydroxyl radical and other oxidant species have been implicated in the depolymerisation of heparan sulphate and proteoglycan core proteins [73, 74] and simultaneously affect protein degradation and cross-linking of type-IV collagen [75], closely involved in the maintenance of the permselective properties of the GBM.

Experimental models of diabetes have demonstrated that elevated circulating levels of glucose and free fatty acids can both be potent activators of NADPH oxidase [76-81]. Further metabolic-mediated increased activation of the RAAS [15] has been recognised as one of the major activators of NADPH oxidase and oxidant species formation [82, 83].

A potent and principle role of NADPH oxidase enzymes in the production of vascular and renal oxidant species has been thoroughly documented in the diabetic kidney [40, 84]. The strong association between NADPH-mediated superoxide anion and hydrogen peroxide production and diabetic nephropathy pathogenesis is evident, with numerous experimental studies reporting enhanced expression of NADPH oxidase subunits in the diabetic renal compartment [80, 85-91].

Of interest and extensive scientific investigation in recent years is the Nox4 subtype, which has been identified as the key source of renal oxidant species driving diabetic nephropathy [91, 92]. Inhibition of Nox4 activity results in decreased oxidative stress and renal tissue damage in experimental models of diabetic nephropathy [78, 93]. Gorin et al. reported attenuation of renal hypertrophy and mesangial expansion following treatment with Nox4 antisense oligonucleotides in streptozotocin-induced diabetic rats [91]. The renoprotective effects of targeting Nox4 have also been demonstrated in a study by Jha et al. in which Nox4 knock-out was associated with prevention of glomerular damage in streptozotocin-induced diabetic mice [78].

Nox1 and Nox2 are also believed to be important players in the pathogenesis of diabetic nephropathy [77]. The overexpression of Nox2 in the kidneys of diabetic mice has been described in a study by Fukuda et al.; in this study, treatment with either angiotensin receptor blockers or ppar-y agonists resulted in inhibition of Nox2 overexpression and a parallel reduction in oxidative stress and renal fibrosis [94]. These findings are consistent with the work of Oudit et al. where treatment with human recombinant angiotensin converting enzyme-2 resulted in Nox2 downregulation and reduction of kidney injury in Akita diabetic mice [95]. A recent study by Nagasu et al. demonstrated an association between increased endothelial Nox2 activity in transgenic diabetic Akita mice and renal injury [77].

Nox2 expression and superoxide production from macrophages [96] has been implicated in DN as advanced oxidation protein products promote inflammation and Nox2 upregulation has been observed in experimental animal model of diabetes [90]. The role of Nox2 in DN is still to be defined as studies in Nox2 deficient mice did not demonstrate a role for Nox2 in DN as observations were confounded by a parallel Nox4 upregulation [88]. Of note, Nox2 inhibition appears an inappropriate target in DN because of the increased susceptibility to infections seen in Nox2-deficient animals [97].

Dual pharmacological inhibition of Nox4 and Nox1 has been shown to successfully reduce oxidative stress and subsequently, renal fibrosis and albuminuria in recent studies of diabetic mice models [93, 98]. However, a recent study suggests pan-inhibition of Nox1, Nox2 and Nox4 provides improved renoprotection in db/db mice compared to dual Nox1/Nox4 inhibitors [99]. Consequently, the Nox family poses a promising therapeutic target in the amelioration of oxidative stress for the prevention and treatment of diabetic nephropathy.

Podocytes are very susceptible to oxidant species-mediated damage. Hyperglycaemia-induced oxidant species results in podocyte dysfunction/damage following the activation of several pathophysiological events such as apoptosis, cell detachment from GBM, podocyte foot process

fusion/effacement, cytoskeleton alterations and reorganisation, and dysregulation of crucial podocyte proteins involved in the regulation of glomerular capillaries permeability.

Different mechanisms have been implicated in podocyte apoptosis [100, 101] in diabetes: autophagy, alteration in cell cycle and proliferation, cell death secondary to alteration in cell-matrix interaction, necrosis and cell-in cell death. Both activation of NADPH oxidase and mitochondrial oxidant species generation have been identified as activators, in podocyte, of pro-apoptotic pathways (p38MAPK and caspase-3) in experimental animal models of diabetes [100, 102, 103]. Increased diabetes-mediated secretion of TGFβ1 has been implicated in podocyte apoptosis via SMAD-7/p38MAPK/caspase-3 activation [104] or Blc2-associated X protein (Bax) expression/translocation in the mitochondria, which in turn results in cytochrome-c release from mitochondria and activation of caspase-3 [100, 104, 105]. Hydrogen peroxide-mediated increase in TGFβ1 expression [89, 106] has been found to fuel NADPH oxidase activation [107] and increases in mitochondria oxidant species production [108], which contribute towards an increase in cellular oxidative stress and secondary apoptosis. Furthermore, increased expression of antioxidant enzymes in transgenic diabetic mice has demonstrated protective properties against diabetes-mediated oxidative stress and parallel podocyte protection in early phases of diabetic nephropathy [109].

Oxidant species have been implicated in podocyte detachment/apoptosis via downregulation of α3β1 integrin [110-112], one of the most important podocyte anchoring receptors on the GBM [113]. Podocytes are indeed found in the urine of patients with diabetes and represent a marker of renal disease progression [114].

Oxidative stress activates [115, 116] Rho-GTPases, which in turn have been linked to podocyte dysfunction, specifically in processes involving cytoskeleton rearrangement and foot process effacement [117].

Diabetes-mediated changes to the mitochondria and the closely connected endoplasmic reticulum (ER)[118] play an important role in diabetic glomerulopathy. As discussed, the mitochondrial

metabolic overload and resulting increased cellular oxidative stress results in ER-stress which leads to the activation of unfolded protein response (UPR)[119]. UPR is a positive cellular response that in its early phase either refolds accumulated unfolded proteins, or degrades unfolded protein by the ubiquitin-proteasome pathway. However, when the unfolded protein and cellular damage exceeds a threshold, chronic and unresolved stress results in a change from an adaptive to pro-apoptotic responses [119].

There is evidence that oxidative stress-mediated ER stress could play a role in diabetic kidney disease [120]. Indeed, hyperglycaemia and the subsequent increased glycation of proteins have been shown to mediate apoptosis partly through increases in ER stress in murine podocytes cultured *in vitro* [121, 122].

Adenosine monophosphate-activated protein kinase (AMPK) is a stress-activated kinase that acts to preserve cell survival under conditions of reduced substrate utilisation. AMPK activation promotes mitochondrial substrate utilisation and ATP generation, in parallel with stimulation of antioxidant gene expression to ensure an optimal redox balance [123]. In diabetes, AMPK is downregulated in the kidney and associated with impaired mitochondrial function [124] and reduced AMPK-mediated inhibition of NADPH oxidase (Nox2) resulting in increased oxidant species production [85, 125]; upregulation/activation of AMPK has been proposed as a potential therapeutic intervention in the diabetic kidney [126].

As mentioned above, oxidative stress mediates extracellular matrix production in the glomeruli. This has been demonstrated in experimental models of glomerular hypertension (Dahl salt sensitive rats)[127], in young spontaneously hypertensive rats [128] and in experimental mouse model of diabetes [129]. Elevated levels of oxidant species stimulate fibronectin mRNA expression, protein synthesis via PKC activation and activation of the transcription factors NFkβ and activator protein-1 (AP-1) both in experimental animal models [130] and in human diabetic glomeruli [131].

Upregulation of heme oxygenase 1 (HO-1), one of the major antioxidant response proteins, has been implicated as a cytoprotective mechanism in the kidney [132-134]; fibronectin expression is

increased in glomeruli of HO-1 deficient animals [135], and bilirubin, a product of the HO-1 metabolism of heme, is known to attenuate TGFβ1-mediated fibronectin expression [136]. Furthermore, Nrf2, a potent transcription factor regulating antioxidant response [137], has been found to act as a transcriptional repressor of TGFβ1, both *in vivo* and *in vitro*, by interacting with the transcription factors c-Jun and SP1 and inhibiting their pro-TGFβ1 effects [80]. A recent report suggested that TGFβ1 may not be necessary for extracellular matrix deposition in patients with diabetic nephropathy [138], and studies have proposed a superoxide-activated ERK-dependent extracellular matrix gene transcription in mesangial cells [139], implicating more of a direct effect of Nrf2/HO-1 axis on fibrosis.

Sulforaphane, an activator of Nrf2, promotes amelioration of diabetic nephropathy in animals through decreased expression of TGF-β1 and connective tissue growth factor (CTGF) [140, 141]. Other Nrf2 activators have also shown a clear protective role in experimental animal models of diabetic kidney disease [142, 143].

The increased oxidant species production in the glomerular microcirculation [144] results in the reduced availability of nitric oxide (due to eNOS uncoupling), resulting in oxidative stress-mediated inflammation, endothelial dysfunction and podocyte detachment from the glomerular capillaries with increased glomerular permeability [15].

In experimental models of type 2 diabetic nephropathy, overexpression of CuZnSOC, a variant of the antioxidant superoxide dismutase enzyme, protects against end organ damage [145]. Furthermore, polymorphisms in manganese-superoxide dismutase, an additional enzyme subtype, are associated with the development of diabetic nephropathy in patients with T1DM [146], supporting an important role of oxidative stress in diabetic kidney disease.

Tubular Compartment in DN

Although the glomerulus has a well-defined role in the pathogenesis of diabetic nephropathy, the tubular compartment is highly affected by diabetes [147]. The tubular interstitial compartment

constitutes up to 90% of renal volume and is composed of the renal tubules, interstitial cells and the renal microvasculature. One third of patients with diabetic nephropathy have minimal glomerular alterations, however marked lesions affecting the tubule-interstitial compartment are present in this patients' group [148]. A stronger correlation between the progression of nephropathy and tubular-interstitial alterations, as compared to glomerular alterations, is widely recognised [148, 149].

Tubular proteinuria is an early marker of DN and precedes microalbuminuria at the glomerulus [150]. Tubular hypertrophy and thickening of the tubular basement membrane are early histological changes associated with diabetic nephropathy. With advanced disease, this manifests with tubular atrophy and tubulointerstitial fibrosis [151].

Oxidative stress in the tubular compartment

Hyperglycaemia affects the tubular structures directly from the tubular cell base-lateral side and, in parallel, the increase in glucose filtration results in an elevated tubular glucose load and exposure. Of interest, diabetes is paralleled by an upregulation of the Na+ coupled energy dependent glucose transporter SGLT2 (localized in the proximal tubuli)[152, 153], the major player in glucose reabsorption in the nephron [154]. The upregulation of SGLT2 and secondary increase in glucose proximal tubule reabsorption results in the activation of the local angiotensin II system and growth factors (e.g. CTGF, TGFβ1) resulting in tubular hypertrophy, increased oxidative stress, tubular cells apoptosis, inflammatory infiltrates, oxidative stress and increased extracellular matrix deposition [155-158].

In physiology, the kidneys receive approximately 25% of cardiac output which potentially could deliver 84 mL/min/100 g tissue of oxygen; of importance, renal oxygen consumption is close to 6.8 mL/min/100 g [159]. Arterial-to-venous (AV) oxygen shunting occurs in the kidneys as a protection against hyperoxia-induced reactive oxygen superoxide production [160-162].

Conversely, diabetic kidneys are susceptible to hypoxia: hyperglycaemia-induced hyperfiltration and the increase in renal blood flow, paralleled by increased oxygen consumption, result in an enhanced arterio-venous oxygen gradient which in turns leads to an increased arterio-venous oxygen shunting and hypoxia [160, 163].

In physiology, the transcription factor hypoxia-inducible factor (HIF-1α) mediates cell adaptation to hypoxia in the renal tubuli by stimulating vasculogenesis and protecting against fibrotic processes through the inhibition of connective tissue growth factor [164, 165]. The diabetic hypoxic kidney is characterised by a lack of compensatory activation of HIF-1α as hyperglycaemia *per se* promotes HIF-1α protease degradation [166, 167] and *via* excess of oxidant species production which has been implicated in HIF-1α destabilization; this is paralleled by progressive inflammatory responses and tubulointerstitial fibrosis [168].

Sources of Oxidative Stress in Diabetes (Fig.3)

Many sources of oxidative stress have been described and implicated in diabetic kidney disease.

Nicotinamide adenine dinucleotide phosphate (NAPDH) oxidase:

NADPH oxidase is formed by the interaction of different enzymes that transport electrons across the cell membrane, reducing oxygen to superoxide. Of these enzymes, the major identified transmembrane subunits are Nox1, Nox2 (gp91phox), Nox3, Nox4, Nox5, and two dual oxidases, Duox1 and Duox2. The catalytic subunits of Nox1-4 depend on the membrane bound p22phox subunit, while the others are independent of p22phox.

Upon stimulation, specific combinations of cytosolic subunits p47phox, p67phox, p40phox, and GTPase Rac1 or Rac2, form a complex that associates with the membrane subunits to form an active NADPH oxidase multi-subunit complex and promote the generation of superoxide [169].

In the kidney, NADPH oxidase has been identified in both the glomerulus (in mesangial cells, endothelial cells and podocytes) and in the tubular compartment [170-176]. Specifically, Nox1, Nox4, p22phox, p47phox, and p67phox are predominantly expressed in mesangial cells, podocytes

express mainly Nox1, 4 and 5, and p22phox [170, 171, 177-181], while Nox1, 2, 4 and 5 and p47phox have been identified in glomerular endothelial cells [78, 169, 179, 182]; Nox5 is also expressed in proximal tubular cells, with Nox4 and Nox1 distributed in the whole tubular compartment [169, 171](**Table 2**).

The role of NADPH oxidase is very important in both the physiological and disease setting where it drives oxidant species production as signalling molecules or oxidative-stress mediated cellular damage respectively [183]. NADPH oxidase is activated by various stimuli such as cellular ligands like growth factors (e.g. epidermal growth factor and TGF-β1), cytokines (e.g. tumour necrosis factor-α), and G protein-coupled receptor agonists (e.g. angiotensin-II, endothelin-1), and physical stimuli (e.g. high glucose, advance glycation product, lipids, mechanical stretch, shear stress)[184-187].

Nox2, highly expressed in lymphomonocytes, may play an important role in inflammatory processes [188], key in the pathophysiology of DN.

Mitochondrial oxidative stress:

One of the major determinants of cellular oxidative stress in renal cells is the excess in cellular glucose uptake. This is driven by the interaction of ambient elevated circulating glucose and the haemodynamic perturbations at the glomerular capillary level, resulting in upregulation of basal glucose transport [17, 39].

Once transported into the cells, glucose undergoes glycolysis to form pyruvate, which is further metabolised in the Krebs cycle to generate nicotinamide (NADH) and flavin adenine dinucleotide ($FADH_2$). These molecules participate as electron donors during oxidative phosphorylation and, in mitochondria, generate adenosine triphosphate (ATP). In conditions of hyperglycaemia (high glucose) and/or mitochondrial dysfunction, the mitochondrial ability to transport electrons is overcome with a subsequent increase in superoxide production [28, 189, 190].

The accumulation of free radicals results in DNA alterations/damage that occurs mainly at the level of mitochondrial deoxyribonucleic acid (DNA), which is deficient in histones and therefore more susceptible to damage. Mitochondrial DNA damage, in turns, leads to further alteration on the respiratory chain resulting in further accumulation of free radicals.

eNOS uncoupling

Nitric oxide synthase (NOS) is expressed, in dimeric form, in most renal cells where all its three major isoforms are represented: inducible NOS (iNOS), neuronal NOS (nNOS) and endothelial NOS (eNOS)[191, 192]. eNOS has been implicated in superoxide production in diabetes when metabolic and haemodynamic perturbations alter the eNOS ability to generate nitric oxide (NO). In diabetes eNOS functionality is affected by the availability of its major substrate L-Arginine and cofactors such as tetrahydrobiopterin (BH_4)[193]. BH_4 is an important mediator of eNOS regulation in diabetes [194], and its reduced availability results in endothelial eNOS uncoupling from its major substrate L-Arginine resulting in inhibition of eNOS-mediated NO formation, with eNOS acquiring the ability to form superoxide and contributing to oxidant species imbalance [195, 196]. Specifically, Satoh et al have documented the uncoupling of eNOS as an additional source of superoxide in isolated glomerular tissues from streptozotocin-induced diabetic rats; restoration of physiological BH_4 concentration was associated with diminished oxidant species generation and improved renal function [197]. These findings support the earlier work in which inhibition of eNOS using L-nitro-arginine methyl ester (L-NAME) prevented renal tissue injury in diabetes [198].

Xanthine oxidase pathway

Xanthine oxidase and xanthine dehydrogenase expression depends closely on cellular content of hydrogen peroxide (e.g. in diseased tissue)[199] and are mainly expressed in epithelial cells. Xanthine oxidase utilizes hypoxanthine or xanthine as a substrate and oxygen as an electron acceptor to generate superoxide and uric acid. In experimental model of diabetes, xanthine

oxidase is abnormally active in kidneys of diabetic rats and has been linked to oxidative stress and the pathophysiology of diabetic nephropathy [200]. Xanthine oxidase is also involved in the synthesis of uric acid, which in turn has a recognised role in diabetic nephropathy [201, 202]; indeed uric acid favours excess production of oxidative stress, drives inflammatory processes and promotes cell death [203]. Uric acid levels have been shown to correlate with cardiovascular and kidney disease [204].

Cyclooxygenase pathway

The cyclooxygenase pathway is important in arachidonic acid metabolism, and is highly expressed in the kidney [205]. Arachidonic acid derives from the cleavage of membrane phospholipids by phospholipase A2 which produces the major substrate for cyclooxygenase (COX) in the synthesis of prostaglandins G2 and, subsequently, prostaglandin H2. This is further metabolised by prostaglandin and thromboxane synthases to produce various prostaglandins and thromboxane A2, known to be important mediators of vascular tone and salt and water balance in the kidney. In mammals two major COX isoforms have been identified: the “constitutive” COX-1 and the inflammatory-mediated COX-2 isoform [205]. COX-1 is expressed in both the glomerular and tubular compartment while COX-2 is mainly expressed at the level of macula densa [206, 207].

COX-2 is a source of free radicals [208] and its increased activity associates with increased oxidant species production and apoptosis in renal cells in culture [209, 210]. Products of COX-2 such as thromboxane and PGE_2 can induce, via the prostaglandin EP1 receptor, NADPH oxidase activity [211, 212] and in turn, oxidant species can induce COX-2 expression [213]. Conversely, PGE2 acting via the prostaglandin EP4 receptor can inhibit free radical production [214, 215].

COX-2 mediated oxidative stress reflects approximately 20-30% of total kidney oxidant species production, and it may play a more important role in ageing as its activity appears to increase with age, as observed in experimental animal models of diabetes.

The Brownlee hypothesis: an unifying theory (Fig. 4)

The pathogenesis underlying diabetic complications has been originally hypothesised by Brownlee who presented a unifying theory in which increased oxidant species formation, as a result of chronic hyperglycaemia, is the single common upstream event driving a cascade of events that result in the development and progression of chronic vascular diabetic complications [30].

Prolonged exposure to high glucose levels induces DN [31] by modulating a variety of different signalling pathways [30]. Chronic hyperglycaemia increases mitochondrial oxidant species production, a central driving force in diabetic nephropathy pathogenesis and a significant factor contributing to accelerated cellular apoptosis [40, 216].

In normal physiological conditions electron transfer through complexes I, III, and IV, in the inner mitochondrial membrane, extrudes protons into the intermembrane space; the derived proton gradient drives the synthesis of ATP through complex V. Conversely, in diabetic condition characterised by elevated intracellular glucose concentration, more glucose is oxidised in the glycolytic and tricarboxylic acid cycle, causing an increase of electron donors, such as NADH and FADH2, into the electron transport chain that results in an increase in gradient across the inner mitochondrial membrane for ATP synthesis. When the gradient reaches a critical threshold, the transfer of electron at the level of complex III is blocked resulting in coenzyme Q to donate electrons to oxygen molecules, resulting in the generation of superoxide [28, 189, 217] which is then degraded by superoxide dismutase to hydrogen peroxide [218].

DNA damage by oxidant species leads to subsequent activation of poly-ADP-ribose polymerase (PARP), a DNA repair enzyme [219]. PARP activation results in the accumulation of ADP-ribose and secondary inhibition of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), a key enzyme in the glycolytic process.

Inhibition of GAPDH leads to the accumulation of precursors in the glycolytic cascade and secondary activation of the pentose, glucosamine, protein kinase C (PKC), and advance glycation

end products (AGE) methylglyoxal pathways, all of which are involved in the pathophysiology of DN [30]. Activation of the above mentioned metabolic pathways subsequently results in dysregulation of numerous cellular signalling molecules involved in inflammation (e.g. nuclear factor-kappa B (NF-kB)), cell response to insult (e.g. p38 mitogen-activated protein kinase (MAPK), Jun N-Terminal kinases [15]), and activators of ER stress and unfolded protein response.

Conversely inhibition of mitochondrial reactive oxygen species has resulted in lack of glucose-induced activation of PKC, formation of advanced glycation end-products, accumulation of sorbitol and activation of the transcription factor NFkβ [189].

The polyol pathway

In normal physiology, a small percentage of glucose is metabolised through the polyol pathway as aldose reductase, a NADPH-dependent enzyme, has a low affinity for glucose. However, increased entry of glucose into cells in hyperglycaemic conditions results in more glucose entering the polyol pathway leading to secondary accumulation of sorbitol in cells, as excess glucose is converted to sorbitol by aldose reductase [30]. Increased sorbitol disrupts cellular osmolality and induces biochemical changes, such as NADP+ generation, which further contributes to increased oxidative stress and non-enzymatic glycation of proteins [216]. Moreover, further oxidation of sorbitol to fructose by the enzyme sorbitol dehydrogenase produces NADH which is believed to exacerbate the inhibition of GAPDH and simultaneously increase cellular levels of triose phosphate [220]. The detrimental consequences of increased activity of the polyol pathway are thought to be most closely associated with reduced concentrations of glutathione, secondary to increased NADPH consumption [221].

Ultimately, the definitive role of the polyol pathway in DN is still an area of debate where no definitive conclusions have been reached [40].

Cellular formation of advanced glycation end products (AGEs)

AGEs are a heterogeneous group of proteins, lipids and nucleic acids, cross-linked with reducing sugars that can determine the activation of cellular signalling proteins involved in increasing oxidative stress, inflammation and cytokine release [222]. Molecules generated from the auto-oxidation of glucose and fructose, such as methylglyoxal, glyoxal and 3-deoxyglucosone, are known as AGE precursors [30, 223]. Thus, hyperglycaemia augments the production of AGEs and raised AGEs concentrations have been reported in the glomeruli of diabetic patients [224]. These molecules are involved in the pathogenesis of DN through multifactorial mechanisms, with evidence suggesting a significant role in diabetes-induced vascular injury [225, 226]. Intracellular protein dysfunction, abnormal extracellular matrix modification and AGE-receptor mediated oxidant species production are the three main mechanisms by which AGEs and their precursors lead to target cell damage [28]. AGE inhibitors have been found to partially reduce the development and progression of DN in experimental models of diabetes, thus highlighting the significance of AGEs in the pathogenesis of DN [227].

The protein kinase C pathway

The PKC family is formed of 11 isoforms, a majority of which are activated by diacylglycerol (DAG), a second messenger found in elevated concentrations in hyperglycaemic intracellular environments [228]. DAG is increased in the diabetic glomeruli and many studies have confirmed the link between hyperglycaemia, direct activation of the diacylglycerol-protein kinase C (DAG-PKC) and the development of cardio-renal vascular disease [229]. Indirect activation of the PKC pathway in hyperglycaemia, through increased polyol pathway and increased AGEs, has also been proposed [230]. Increased PKC activation (mainly of the β and δ isoforms) can lead to disruptions and altered expression of many intercellular proteins, namely eNOS, endothelin-1, vascular endothelial growth factor and TFG-β1 [216]. The effects of this vary from basement membrane

thickening, to vascular permeability alterations to pro-inflammatory gene expression. Specific β-isoform PKC inhibitors have been shown to counter enhanced mesangial expansion in the glomerulus [231]. Of interest, other isoforms such us PKC-α and PKC-ε have been proposed as protective in the pathophysiology of DN [232].

The hexosamine pathway

Hyperglycaemic conditions cause excess glucose to be shunted into the hexosamine metabolic pathway, which presents an additional pathway leading to the manifestations of diabetic complications [233]. An increase in activity of this pathway causes increased levels of fructose-6-phosphate with subsequently elevated expression of TGF-α, TGF-β1 and plasminogen activator inhibitor 1 (PAI-1)[217, 233]. This has negative effects leading to increased extracellular matrix expression and accumulation at the tissue level [30].

Novel perspectives on oxidant species in diabetic nephropathy: mitochondrial hormesis

The hypothesis that oxidant species are a major driver in the pathophysiology of chronic diabetic vascular complications and DN, depicted by Brownlee, has been challenged by negative results obtained in antioxidant-based clinical trials [40].

Following these observations, a new theory of "mitochondrial hormesis" has been proposed [234]: mitochondrial hormesis describes the concept that oxidant species, not only causes oxidative stress if elevated, but may function, at physiological concentration, as signalling molecules that promote health; therefore mitochondrial superoxide production could be considered as an indicator of healthy mitochondria and physiologic oxidative phosphorylation.

Research in experimental animal models of diabetes (STZ-induced diabetic Akita-mice) has demonstrated a reduction in the levels of renal mitochondrial superoxide, secondary to diabetes-mediated alteration of mitochondrial respiration [124]. Based on these findings, genetic or

pharmacological correction of mitochondrial respiration should confer a degree of renoprotection in mouse models of tubulointerstitial fibrosis [235].

Studies have highlighted the need for a more targeted antioxidant approach towards specific cell compartments as, in addition to mitochondrial oxidant species production, cytosolic and other non-mitochondrial sources of oxidant species may also play a role in the pathophysiology of DN [236]. Moreover, studies in humans have demonstrated that leukocytes, from patients with diabetes and DN, have a reduced maximal respiration and reserve capacity (when compared to non-diabetic controls) suggesting that the diabetic environment does manifest with alteration in ATP-linked respiration, low reserve capacity and mitochondrial damage [237, 238].

The Brownlee [28] and "mitochondrial hormesis" [234] hypotheses, apparently in contrast, could represent the same phenomena but at different stages of disease progression. One could speculate that in the initial phase of diabetes, the hyperglycaemic/haemodynamic insult to the kidney drives excess oxidant species production. This may gradually lead to secondary impairment of the mitochondria cellular respiratory machinery and consequently, result in altered respiration with reduction in pyruvate oxidation, reduced oxidant species production and cell death. The reduction in pyruvate oxidation in tricarboxylic acid cycle has been attributed to an increase in pyruvate dehydrogenase phosphorylation (as found in diabetic kidney)[124], known to inhibit pyruvate uptake into mitochondria. Of interest a recent report suggests that activation of pyruvate kinase M2, improves mitochondrial dysfunction by increasing mitochondrial metabolism and mitochondrial mass [239].

The safeguarding of an adequate number of healthy mitochondria in disease seems to be critical for both cell survival and for the preservation of a "balanced" level of cellular oxidant species (**Fig. 5**).

Therapeutic strategies in diabetic nephropathy

Current treatment strategies

The central concept in treating diabetic complications is prevention of its known risk factors and this has largely been influenced by findings from major landmark clinical trials. The Diabetes Control and Complications Trial (DCCT) demonstrated that intensive glycaemic control in T1DM had major benefits in preventing GFR decline [31] and the development and progression of proteinuria [22]. Furthermore, these patients experienced a long lasting reduction of approximately 40% in the risk for the development of micro-albuminuria, for up to 7-8 years after the trial [240].

In patients with T2DM, the United Kingdom Prospective Diabetes Study (UKPDS) found a 30% reduction in the incidence of micro-albuminuria for the intensively treated group with better diabetes control [241]. Furthermore, the UKPDS also identified that a reduction of systolic blood pressure reduced the development of micro-albuminuria by 29% [242]. Blockers of the RAAS have been shown to slow down the progression to ESRD, independently of their antihypertensive effect; RAAS blockade significantly attenuates oxidant species production through the inhibition of angiotensin II-mediated NADPH oxidase activation. However, complete oxidant species production suppression is not completely achieved and alternative sources of oxidant species, such as the mitochondria, might still play a role in DN progression [243].

The role of antioxidant therapy: results from clinical trials

Given the prominent role of oxidant species in the development of diabetic nephropathy, anti-oxidant therapies have been tested as a promising and exciting avenue of investigation and research in the progression of diabetic kidney disease and other chronic diabetic complications. Theoretically, the ideal anti-oxidant therapy would be specific, have minimal side effects and effectively target all identified oxidant species pathways.

To date many trials have been conducted but the clinical observations in humans have not matched the promising observations obtained in pre-clinical animal models [244].

In studies in patients with diabetes, administration of Vitamin E, an antioxidant acting as a peroxyl radical scavenger, failed to show beneficial effect on vascular chronic complications [245]; further antioxidant supplementation has not shown any definitive benefit on endothelial dysfunction and specifically, on renal outcomes [246, 247].

Conversely some studies investigating vitamin E oral administration normalized hyperfiltration in patients with type 1 diabetes [248], and antioxidant combination therapies of vitamins E and C seems to confer some degree of renoprotection in patients with type 2 diabetes, though results are not conclusive [247]. Benfotiamine, a synthetic S-acyl derivative of thiamine (vitamin B1) with antioxidant properties, has been shown to reduce albuminuria in patients with type 2 diabetes [249].

More recently bardoxolone-methyl, an activator of the antioxidant transcription factor Nrf2 with reported antioxidant and anti-inflammatory properties, resulted in increased albuminuria paralleled by hyperfiltration and increased risk of death in patients with advanced renal disease [250]. Promising results have recently emerged in experimental animal models of diabetes investigating the effectiveness of a novel analogue of the Nrf2 agonist bardoxolone methyl [251].

Three months administration of silymarin, a unique flavonoid complex derived from the milk thistle plant with antioxidant and anti-inflammatory properties, was paralleled with a reduction in urinary excretion of albumin, TNF-α, and malondialdehyde in patients with diabetic nephropathy [252].

From these series of studies, it seems that most attempt in reducing oxidative stress in humans with dietary antioxidant therapy do not translate in organ protection; the reasons might be different.

Oxidative stress may not be only process involved in the pathogenesis of a disease. As an example, in DN, pathophysiological processes such as inflammation, activation of apoptotic pathways might

be partly unrelated to the increase in oxidant species, and therefore might not be completely targeted by antioxidant therapy. Bioavailability could also be an issue: many of the compounds utilised are susceptible to auto-oxidation in the gut, have limited absorption and can be rapidly metabolised by the gut microbiota reducing their bioavailability [253, 254]; in this respect, there might be a patient susceptibility for a response to antioxidant therapy. Future work might have to consider different, more stable formulation of antioxidants to plan new clinical research trials.

Novel therapeutic approaches

There has been growing interest in targeting uric acid as an antioxidant treatment for diabetic nephropathy and current ongoing trials in humans are testing the role of allopurinol as a nephroprotective agent in patients with T1DM [255].

The NOX1/NOX4 inhibitor GKT137831 (Genkyotex) showed considerable promise in its phase-I trial, but failed to reduce albuminuria in patients with diabetes and kidney disease (https://www.genkyotex.com/en/pipeline/gkt831); further studies are on the way to dissect the potential positioning of this molecule in clinical medicine.

Pentoxifylline, a methylxanthine derivate and nonspecific phosphodiesterase inhibitor clinically used to treat patients with occlusive peripheral vascular disorders, which retains anti-inflammatory, anti-fibrotic anti-oxidant properties [256] has revealed a renoprotective role in patients with diabetes. Additional studies are needed to confirm these promising results in larger clinical trials [257].

Concluding remarks and future perspectives

We believe that there is strong experimental evidence that oxidative stress is involved in the pathophysiology of DN. The overall failure of clinical trials using antioxidant for the treatment of DN should be carefully assessed before coming to definitive conclusions.

We will need to understand better the role of oxidative stress in either physiology and disease before we can securely test novel antioxidant treatments. Key understanding to be answered in future studies is the role of specific oxidant species within each cellular compartment both in physiology and disease conditions; further there is building evidence that oxidant species have a clear role in cell signaling [258], and therefore putative treatments targeting excess oxidant species will have to "modulate" rather than inhibit cellular oxidative status.

The role of oxidant species as pro-oxidant and/or activators/inhibitors of cellular signalling pathways is likely to be different in different diseases setting (e.g. acute *versus* chronic) and could also change as diseases progress and evolve with time.

Careful understanding of the mechanisms of any new molecule able to modulate cellular oxidative stress will have to be a prerequisite for its translation in the clinical setting. The altered dynamics of oxidant species in cells and tissues in diseases, makes the search for new antioxidant therapy challenging. Challenges often bring new discoveries; hopefully research targeting antioxidant specific interventions to ameliorate DN or disease in general will be translated to humans in the next few years.

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Figures:

Fig. 1: Metabolic-haemodynamic interaction in DN: Schematic representation of interaction between metabolic and haemodynamic factors contributing to diabetic nephropathy.

Fig. 2: Structural schematic of normal and diabetic glomerulus. Normal (left) and diabetic (right) glomeruli. The diabetic glomeruli is characterised by podocyte apoptosis and detachment, thickening of the GBM, endothelial cell apoptosis, and mesangial expansion).

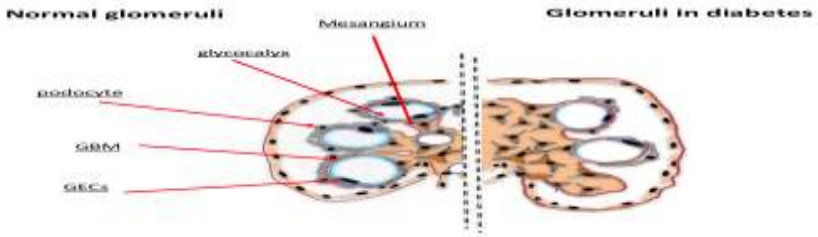

Fig. 3: **Sources of oxidative stress in diabetes.** NADPH: nicotinamide adenine dinucleotide phosphate; eNOS: endothelial nitric oxide synthase; COX: cyclooxygenase.

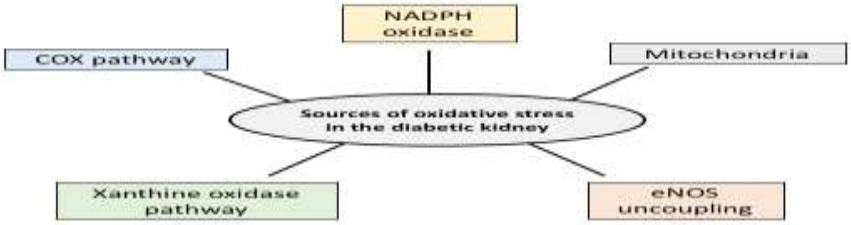

Fig. 4: Pathophysiology of diabetic nephropathy. Schematic overview of the signalling cascade induced by hyperglycaemia-mediated activation of the four key pathways underlying the pathogenesis of diabetic nephropathy; the polyol pathway, the advanced glycation end products pathway, the hexosamine pathway and the protein kinase C pathway. (PARP - poly-ADP-ribose polymerase; GAPDH - glyceraldehyde phosphate dehydrogenase; MAPK - mitogen activated protein kinase).

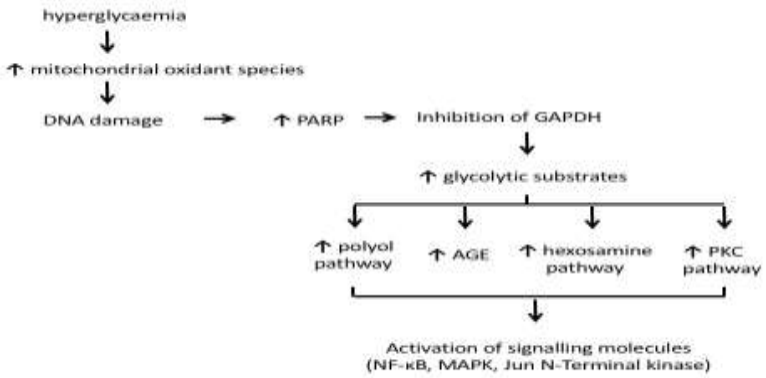

Fig. 5: Mitochondrial dysfunction in diabetes. Excess glucose entry in the cell will initially determine an increase oxidative stress (left image); this scenario might change in later stages of the disease characterised by mainly mitochondrial dysfunction and cell death, a scenario paralleled by a reduction in free radical production by mitochondria (right image) (TCA: tricarboxylic acid; ETC: electron transport chain; O_{2}-: superoxide).

Tables:

Table 1: Stages of diabetic nephropathy

Stage of Diabetic Nephropathy	Description
Stage 1: Glomerular Hyperfiltration	Early hyperfunction and hypertrophy
Stage 2: Silent Stage	Glomerular lesions without clinical disease
Stage 3: Incipient Nephropathy with Microalbuminuria	Urine albumin excretion (UAE) 30-300mg/day
Stage 4: Overt Nephropathy	Urine albumin excretion (UAE) >300mg/day
Stage 5: End-Stage Renal Disease	Major loss of kidney function, requires dialysis

Table 2: Distribution of NADPH oxidase subunits in renal cells

Anatomical site	NADPH component	References
Renal cortex	Nox1, Nox2, Nox4 $p22^{phox}$, $p47^{phox}$, $p67^{phox}$	[176]
Renal vessels	Nox-2, $p22^{phox}$	[182]
Microvascular glomerular endothelial cells	Nox1, Nox2, Nox4, Nox5, $p47^{phox}$	[78, 169, 179, 182]
Glomeruli	Nox1, Nox 2, Nox4, Nox5, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$,	[177, 259, 260] [78, 169, 179, 182]
Mesangial cells	Nox1, Nox2, Nox4, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$	[170, 173, 261]

Podocytes	Nox1, Nox4, Nox5, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$	[170, 171, 177-181]
Interstitial fibroblast	$p22^{phox}$	[177]
Proximal tubule	Nox5, $p22^{phox}$	[169, 171, 262]
Thick ascending limb	Nox1, Nox4, Nox2, $p40^{phox}$, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$	[169, 179, 263]
Macula densa	$p22^{phox}$, $p47^{phox}$, $p67^{phox}$	[179]
Distal convolute tubule	Nox1, Nox4, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$	[169, 179]
Cortical collecting duct	Nox1, Nox4, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$	[169, 179]
Medullary collecting duct	Nox1, Nox4, $p22^{phox}$, $p47^{phox}$, $p67^{phox}$	[169, 179]

Highlights

- Experimental models suggest an important role for oxidative stress in the pathophysiology of diabetic nephropathy.
- Clinical trials with antioxidant therapy have not delivered convincing results.
- Free radicals are important in cell physiology and treatments targeting oxidative stress in disease conditions should consider that complete inhibition of reactive oxygen species formation is not beneficial.
- Targeting oxidative stress may require different strategies in different phases (early, advanced) of chronic diseases.